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strain overexpressing *patS* (10).

We propose a model in which a processed PatS peptide, originating from differentiating proheterocysts, diffuses along the filament's contiguous periplasmic space and is taken up by neighboring cells, creating a gradient of inhibitory signal. The intracellular target of PatS signaling is unknown, but components of a phosphorelay such as that found in *Bacillus* (13) are likely candidates.

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- pAM1697 contains *patS* that was amplified by PCR and cloned into pPet1 (W. J. Bulkema and R. Hasselton, personal communication), which contains the *patS* promoter. pAM1714 contains the *P_{patS}-patS* fragment from pAM1697 in pAM504 (19). pAM1716 contains *patS* in the reverse orientation. Two independent clones for each construct were tested, and they produced similar results.
- pAM1882 DNA (1 µg) was incubated in a final volume of 100 µl of 0.4 M hydronylamine-HCl in buffer [50 mM sodium phosphate (pH 6.0) and 0.9 mM EDTA] at 65°C for 80 min [H. C. Lee, Y. P. Tsung, Y. S. Tu, C. P. Yu, *J. Biol. Chem.* 270, 99 (1995)]. After dialyzing the DNA against TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] overnight, 2 µl was used to transform *Escherichia coli* conjugal donor strain AM1359 (strain DH108 containing pRL223 and pRL443) [J. Elhal, A. Veprikov, A. M. Muro-Pastor, E. Flores, C. P. Wolk, *J. Bacteriol.* 179, 1998 (1997)]. Several thousand transformant colonies were collected and used for conjugation with *Anabaena* PCC 7120. After incubating for 10 days on BG-11, plates containing neomycin (25 µg/ml), the four best-growing Het⁺ exconjugants were selected for plasmid isolation and DNA sequencing.
- In-frame (pAM1899) and out-of-frame (pAM1860) *patS-lacZ* translational fusions were made by ligating *patS* fragments to *lacZ*. *Anabaena* PCC 7120 exconjugants AMC446 (pAM1899) and AMC448 (pAM1860) were induced in BG-11, for 0, 6, 14, 18, 27, and 48 hours and harvested for β-Gal assays. Cells were lysed and assayed as previously described [M. R. Schaefer and S. S. Golden, *J. Bacteriol.* 171, 2973 (1989)], except that the filaments were frozen at -85°C before processing. β-Gal was measured and expressed as specific activity (nanomoles of o-nitrophenyl-β-D-galactopyranoside per minute per milligram of protein).
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- AMC451 was made by double recombination with suicide plasmid pAM1702 (Fig. 2A) as previously described (19). pAM1702 contains *patS*-flanking sequences and an *Ω* Sp/Smr cassette (conferring spectinomycin and streptomycin resistance) in *sacB*-con-
- taining suicide vector pRL278 [T. A. Black, Y. Cai, C. P. Wolk, *Mol. Microbiol.* 8, 77 (1993)]. The *Ω* Sp/Smr cassette replaces a 381-bp Eco RV-Sca I fragment containing the entire *patS* gene. Four independent isolates with identical phenotypes were obtained after selection on media containing sucrose (5%), spectinomycin (2 µg/ml), and streptomycin (2 µg/ml). The structure of the deletion was confirmed by Southern (DNA) blot analysis.
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- Details of plasmid constructions are available from the authors. Subclones were from pAM1035, which contains *patS* on a 3.3-kb Bam HI-Cla I fragment that was cloned into the same sites of pBluescript II KS(-). pAM1882, pAM1687, and pAM1835 contain restriction fragments in the conjugal shuttle vector pAM504 (19). pAM1685 contains *patS* that was amplified by PCR and cloned into pAM504. pAM1686 contains the same insert cloned into pAM505 (identical to pAM504 except that the Bam HI and Sac I sites are reversed). pAM1691 and pAM1695 contain *patS* cloned into pAM743, which contains the *Anabaena* PCC 7120 *glnA* promoter from pAM658 (20) in pAM504. The sequences of all inserts that were generated by PCR were confirmed after subcloning.
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- The *patS-gfp* transcriptional fusion in pAM1951 was made by first cloning a fragment containing 724 bp upstream of *patS* into pKEN2-GFPmut2 (17) to make pAM1877. A fragment containing *patS-gfp* from pAM1877 was ligated into pAM505 to make pAM1951. *gfp* from pKEN2-GFPmut2 was cloned into pAM542 (20), which contains *P_{gfp}* on a shuttle vector, to make pAM1954. pAM1956 contains promoterless *gfp* from pKEN2-GFPmut2 inserted into pAM505.
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Binding of Hepatitis C Virus to CD81

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Chronic hepatitis C virus (HCV) infection occurs in about 3 percent of the world's population and is a major cause of liver disease. HCV infection is also associated with cryoglobulinemia, a B lymphocyte proliferative disorder. Virus tropism is controversial, and the mechanisms of cell entry remain unknown. The HCV envelope protein E2 binds human CD81, a tetraspanin expressed on various cell types including hepatocytes and B lymphocytes. Binding of E2 was mapped to the major extracellular loop of CD81. Recombinant molecules containing this loop bound HCV and antibodies that neutralize HCV infection in vivo inhibited virus binding to CD81 in vitro.

HCV is a positive strand RNA virus of the flaviviridae family (1) chronically infecting about 170 million persons worldwide (2). Chronic HCV infection results in liver diseases (hepatitis, cirrhosis, and hepatocellular carcinoma) in a sizable fraction of cases (3). Infection with HCV is also associated with most cases of type II and type III cryoglobulinemia, B lymphocyte proliferative disorders characterized by polyclonal B cell activation and autoantibody production (4). The complete HCV sequence has been available since 1989 (5); however, progress in understanding the viral life cycle

has been hampered by the lack of virus culture systems in vitro. Although hepatocytes and B lymphocytes are thought to be infected by HCV (1), there is no consensus on viral tropism, and the cellular receptor for the virus has not been identified.

We have shown previously that a recombinant form of the major envelope protein (E2) of HCV binds with high affinity to human lymphoma and hepatocarcinoma cell lines, whereas it does not bind to mouse cells (6). Furthermore, in chimpanzees vaccinated with recombinant E1 and E2 envelope proteins, protection from homologous HCV challenge correlated with the presence of antibodies capable of inhibiting the binding of E2 to human cells (6). These results suggested that E2 may be responsible for binding of HCV to target cells.

To identify the E2-binding molecule on human cells, we prepared a cDNA expression

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Exhibit B

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library from A2R cells, a subclone of the human T cell lymphoma Molt-4, which exhibited high E2-binding capacity (7). This library was screened by transient transfection of a mouse fibroblast cell line (WOP) (8) with E2 as a probe (9). This approach resulted in the identification of a cDNA clone that conferred E2-binding capacity to WOP cells upon transient transfection. This clone contains an insert of 0.9 kb encoding human CD81. This widely expressed 25-kD molecule is a member of the tetraspanin superfamily (10), which includes cell surface proteins that span the membrane four times, forming two extracellular loops. The intracellular and transmembrane domains of CD81 are highly conserved among different species, whereas the major extracellular loop is quite diverse (Fig. 1). The major loop is highly conserved in humans and chimpanzees, which are the only known species permissive to HCV infection (1) and whose cells bind HCV E2 (6).

To confirm that CD81 was the human cell surface molecule binding HCV E2, we used recombinant E2 and antibodies to CD81 (anti-CD81) to assess this interaction. Recombinant E2 competitively inhibited the binding of anti-CD81 to Epstein-Barr virus (EBV)-transformed B cell lines (EBV-B cells) (Fig. 2A). In addition, E2 reacted in protein immunoblots with anti-CD81-precipitated material (Fig. 2B). CD81 on fresh lymphocytes and hepatocytes is also capable of binding E2, as demonstrated by immunohistochemical staining with biotin-labeled E2 (11).

Because of the lack of HCV culture assays in vitro, we developed alternative methods to demonstrate that CD81 interacts with HCV. We prepared a recombinant fusion protein (Fig. 3A) expressing the major extracellular loop (EC2) (amino acid residues 113 to 201) of human or mouse CD81 fused to the COOH-terminal end of thioredoxin (TRX-EC2) (12). The proteins containing the human, but not the mouse, loop bound to E2 in protein immunoblot (13) and in solution as shown by inhibition of binding of E2 to human cells (Fig. 3B). To assess virus binding, we attached human or mouse TRX-EC2 proteins to polystyrene beads and incubated them with an infectious plasma containing known amounts of viral RNA molecules. After washing, the amount of bead-associated virus was measured by quantitative reverse transcription polymerase chain reaction (RT-PCR) (14). Beads coated with human TRX-EC2, but not with mouse TRX-EC2, bound HCV in a concentration-dependent fashion (15) (Fig. 3C). Preincubation of beads with anti-CD81 inhibited virus binding. Furthermore, preincubation of the infectious plasma with sera from chimpanzees that were protected from homologous HCV challenge by vaccination with E1 and E2 envelope proteins (16) inhibited HCV binding to CD81 (Fig. 3D). In contrast, sera from vaccinated but nonprotected chimpanzees, although containing anti-E2, were not inhibitory

(Fig. 3D). These results demonstrate that anti-E2 antibodies, which are capable of neutralizing HCV infection in vivo, can inhibit the binding of HCV to CD81 in vitro, supporting the idea that CD81-E2 interaction is relevant to infection.

Our data demonstrate that human CD81 is sufficient for binding not only E2 but also HCV particles. Given the wide distribution of CD81 (10), these results imply that HCV can bind to a variety of cells other than hepatocytes. Consistent with this finding, HCV RNA has

been found in T and B lymphocytes and monocytes (17). Whether virus binding to CD81 is followed by entry and infection in all cell types is not clear, because it is possible that additional factors are required for HCV fusion or infectivity.

CD81 participates in different molecular complexes on different cell types, a fact that may affect its capacity to mediate HCV attachment or to deliver signals to target cells. For instance, on epithelial and hematopoietic cells, CD81 associates with integrins (18), whereas

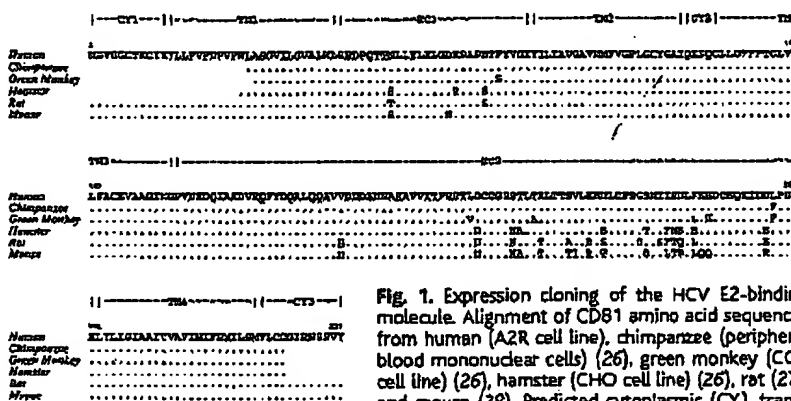


Fig. 1. Expression cloning of the HCV E2-binding molecule. Alignment of CD81 amino acid sequences from human (A2R cell line), chimpanzee (peripheral blood mononuclear cells) (26), green monkey (COS cell line) (26), hamster (CHO cell line) (26), rat (27), and mouse (28). Predicted cytoplasmic (CY), transmembrane (TM), and extracellular (EC) domains are

indicated according to (10). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Fig. 2. Interaction between recombinant E2 and CD81. (A) Dose-dependent inhibition of anti-CD81 binding to B cells by recombinant E2. EBV-B cells were incubated with increasing concentrations of recombinant E2 for 1 hour at 4°C and then stained with an mAb to CD81 (clone JS-81, Pharmingen). The data are expressed as percentage of inhibition of mean fluorescence intensity (6). Preincubation with E2 did not inhibit binding of an anti-major histocompatibility complex class I (clone W6/32) (29). (B) E2 detects, on protein immunoblot, the 25-kD protein immunoprecipitated by anti-CD81.

A membrane protein extract (about 300 µg) prepared from the A2R cell line was solubilized with 8 mM 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfonate in PBS (pH 7.4) and incubated with 10 µg of recombinant E2 (lanes 2 and 3), 20 µg of anti-CD81 (lane 4), or 20 µg of a control antibody (anti-human CD9; ATCC) (lane 5). After incubation for 2 hours at 4°C, the samples were immunoprecipitated with chimpanzee antisera to E2 (lane 2), chimpanzee preimmune sera (lane 3), or goat anti-mouse IgG (lanes 4 and 5) bound to protein A-Sepharose (CL-4B; Pharmacia). The precipitated samples were eluted in Laemmli buffer, separated by SDS-PAGE under nonreducing conditions, and transferred to a PVDF (polyvinylidene difluoride) membrane by electroblotting. The blots were then probed with recombinant E2 (1 µg/ml) overnight followed by a 2-hour incubation with an mAb to E2 (mAb 291). Visualization of the immunoprecipitated proteins detected by E2 was performed with a peroxidase-conjugated polyclonal anti-mouse IgG (Amersham). As a positive control, a portion of the total membrane extract was also loaded on the gel (lane 1). The mobilities of molecular mass (in kilodaltons) markers are indicated at left. The 25-kD CD81 is immunoprecipitated directly by anti-CD81 or indirectly by a combination of E2 and anti-E2 as shown in lanes 4 and 2, respectively. The high molecular mass bands observed in all lanes (including negative control lane) probably represent cross-reaction of the secondary antibody, detection of E2 precipitated by chimpanzee antisera to E2 subsequently recognized by anti-E2 mAb (lane 2), or detection of mAb to CD81 (lane 4) or mAb to CD9 (lane 5) by the secondary anti-mouse.

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PAGE) in nonreducing conditions. After electroblotting, the PVDF membrane (Millipore) was probed overnight with recombinant E2 (1 µg/ml) at room temperature, incubation with an mAb to E2 (clone 29 1A2) was followed by chemiluminescent detection with a peroxidase-conjugated polyclonal anti-mouse IgG (Amersham).

Polystyrene beads (1/4-inch diameter) (Pierce) were coated overnight with purified EC2 recombinant protein in citrate buffer (pH 4) at room temperature. After saturation for 1 hour with 2% bovine serum albumin in 50 mM Tris-Cl (pH 8), 1 mM EDTA, and 100 mM NaCl (TEN) buffer, each bead was incubated at 37°C for 2 hours in 200 µl of TEN-diluted infectious chimp plasma containing 5×10^5 HCV RNA molecules. For inhibition experiments, the EC2-coated polystyrene beads were incubated with purified mAbs (50 µg/ml) for 1 hour at room temperature before incubation with the virus. Each bead was washed five times with 15 ml of TEN buffer in an automated washer (Abbot, Wiesbaden, Germany), and viral RNA was extracted with the Viral Extraction Kit (Qiagen). RNA (8 ml) was reverse transcribed at 42°C for 90 min in 20 µl of buffer A (Taq Man; Perkin-Elmer) containing 100 pmol of the HCV antisense primer CCGTTCGCCAGACCACTATG, 40 U of RNasin (ribonuclease inhibitor) (Promega, Madison, WI), deoxynucleoside triphosphates (dNTPs) (250 µM), MgCl₂ (25 mM), and 10 U of Moloney murine leukemia virus reverse transcriptase (Boehringer). cDNA (20 µl) was amplified with a Perkin-Elmer ABI 7700 Sequence Detection System (45 cycles) in 50 µl of buffer A containing 100 pmol of the HCV sense primer TCTTCACGCCAGCAAGCCTCTA, 5 pmol of the fluorescent detection probe 5'-(FAM)TCAGTCTCGTGCAGCTCCAGGA(TAMRA) (FAM is carboxyfluorescein amino-modified oligo and TAMRA is tetramethylrhodamine amino-modified oligo), dNTPs (300 µM), and 1.25 U of Taq Gold (Perkin-Elmer). All reactions were quantified with HCV (genotype 1a)-infected plasma (branched DNA test of 30 meq/ml) to generate a standard curve. Sequence Detector Software from Perkin-Elmer has been described previously (25). To evaluate virus binding to cells, we made human CD81⁺ mouse stable transfectants (NIH 3T3) that bound E2. However, we consistently failed to measure substantial virus attachment to the cell surface by PCR because of high background inherent to the technique.

15. In our assay, we captured only enveloped RNA molecules. The highest available concentration of human TRX-EC2 for coating beads was 100 µg/ml. At this concentration, about 7% of HCV input was bound by the beads. Using TRX-EC2 (100 µg/ml) and increasing numbers of beads, we captured about 10% of the HCV input. In terms of RNA molecules, further demonstrating that HCV binding is dependent on the CD81 concentration. Moreover, our experience with antibody (from mouse, chimp, or human)-coated beads has shown that the percentage of HCV that can be captured is negligible, further proving that CD81 is indeed a very effective binder of HCV particles.
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TNF- α Induction of CD44-Mediated Leukocyte Adhesion by Sulfation

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Regulation of cell adhesion is important for immune system function. CD44 is a tightly regulated cell adhesion molecule present on leukocytes and implicated in their attachment to endothelium during an inflammatory immune response. The proinflammatory cytokine tumor necrosis factor- α , but not interferon- γ , was found to convert CD44 from its inactive, nonbinding form to its active form by inducing the sulfation of CD44. This posttranslational modification was required for CD44-mediated binding to the extracellular matrix component hyaluronan and to vascular endothelial cells. Sulfation is thus a potential means of regulating CD44-mediated leukocyte adhesion at inflammatory sites.

During an immune response, activated leukocytes leave the circulation and enter the tissues. Leukocyte migration and extravasation is a multistep process involving the rolling and adhesion of leukocytes to the endothelium and their subsequent diapedesis to the inflammatory site (1). Activated T cells can bind the extracellular matrix component hyaluronan, and this

CD44-mediated interaction has been implicated in the rolling and extravasation of lymphocytes at inflammatory sites (2). CD44 is normally present on leukocytes in an inactive state that cannot bind hyaluronan but can be converted to an active state upon appropriate stimulation, such as activation by antigen or cytokines (3, 4). However, the molecular mechanism for this conversion is poorly understood. The binding ability of CD44 has been shown to be affected by three types of posttranslational modification: N- and O-linked glycosylation and glycosaminoglycan addition (4, 5). Here, we show that sulfation is an additional posttranslational mechanism that can convert inactive CD44 to its active, adhesive form and that this mechanism is induced by the proinflammatory cytokine tumor necrosis factor- α (TNF- α).

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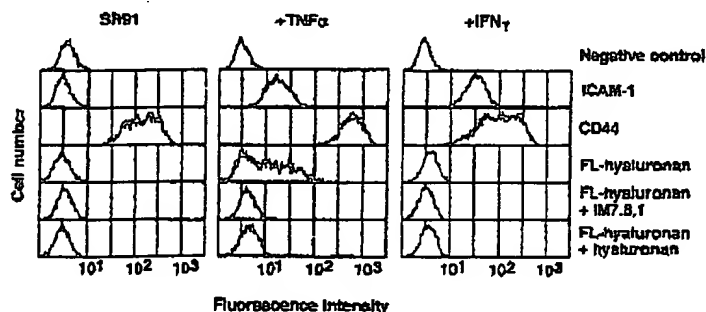


Fig. 1. Expression of CD44 and ICAM-1 and FL-hyaluronan binding ability on untreated, TNF- α -treated, or IFN- γ -treated SR91 cells by flow cytometry. Cells were preincubated with the CD44 mAb IM7.8.1, or with unlabeled hyaluronan, to block binding. Unlabeled SR91 cells were the negative control.